Characterizing Factors Associated with Differences in FGF19 Blood Levels and Synthesis in Patients with Primary Bile Acid Diarrhea

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Abbreviations: ASBT; apical sodium-dependent bile acid transporter; BA, bile acid; BAD, bile acid diarrhea; C4, 7α-hydroxy-4-cholesten-3-one; CDCA, chenodeoxycholic acid; FGF19, fibroblast growth factor 19; FXR, farnesoid X receptor; GCDCA, glyco-chenodeoxycholic acid; IBABP, ileal bile acid binding protein; ID, idiopathic diarrhea; OST, organic solute transporter; SeHCAT, ^{75}Se-homocholic acid taurine; SNP, single nucleotide polymorphism; UTR, untranslated region.

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ABSTRACT

INTRODUCTION: Chronic diarrhea caused by primary bile acid diarrhea (PBAD) is a common condition. We have previously shown PBAD is associated with low fasting serum levels of the ileal hormone, fibroblast growth factor 19 (FGF19). FGF19 is a negative regulator of hepatic bile acid synthesis and is stimulated by farnesoid X receptor agonists, which produce symptomatic improvement in PBAD. We aimed to assess possible causes for low serum FGF19 in patients with PBAD.

METHODS: Patients with PBAD, defined by reduced ^{75}Se-labelled homocholic acid taurine (SeHCAT) retention, and idiopathic diarrhea controls had measurements of fasting lipids and fasting/post-prandial FGF19 serum profiles. Specific functional variants in candidate genes were investigated in exploratory studies. In further groups, basal and bile acid-stimulated transcript expression was determined in ileal biopsies and explant cultures by quantitative-PCR.

RESULTS: FGF19 profiles in PBAD patients included low fasting and meal-stimulated responses, which were both strongly correlated with SeHCAT. A subgroup of 30% of PBAD patients had fasting hypertriglyceridemia and higher FGF19. No clear significant differences were found for any genetic variant but there were borderline associations with *FGFR4* and *KLB*. SeHCAT retention significantly correlated with the basal ileal transcript expression of FGF19 (r_s =0.59, p=0.03) and ASBT (r_s=0.49, p=0.04), and also with the degree of stimulation by chenodeoxycholic acid at 6h for transcripts of FGF19 (median 184-fold, r_s=0.50, p=0.02) and IBABP (median 2.2-fold, r_s=0.47, p=0.04). Median stimulation of FGF19 was lower in patients with SeHCAT retention <10% (p=0.01).

DISCUSSION: These studies demonstrate a complex, multifactorial etiology of PBAD, including impairments in ileal FGF19 expression and responsiveness.

Keywords: Bile acid malabsorption; functional gastro-intestinal disorders; IBS-D

(257 words)

Study Highlights

1. WHAT IS CURRENT KNOWLEDGE

- Chronic diarrhea due to bile acids is common but often unrecognized
- Reduced median serum FGF19 has been shown in patients with primary bile acid diarrhea
- The cause of reduced FGF19 is currently unknown

2. WHAT IS NEW HERE

- Hypertriglyceridemia is found in about 30% of patients with bile acid diarrhea who have normal FGF19
- Patterns of serum FGF19 include low fasting and meal-stimulated levels
- No major genetic differences in candidate genes have been identified
- Ileal FGF19 transcript levels are significantly reduced in both basal conditions and after bile acid stimulation.

INTRODUCTION

Bile acid diarrhea (BAD) is a poorly recognized cause of chronic diarrhea and other symptoms such as urgency and fecal incontinence which may otherwise be diagnosed as the irritable bowel syndrome (IBS). (1-4) Systematic reviews have suggested that up to 30% of diarrhea-predominant IBS patients, including those meeting Rome III IBS criteria, could have primary bile acid diarrhea (PBAD). These studies used data from abnormal ^{75}SeHCAT tests, with patients having reduced 7d retention of this labelled bile acid and no obvious cause for bile acid malabsorption. (2,5-7) SeHCAT values of 10-15% are considered to be mild, 5-10% are moderate, and less than 5% are severe abnormalities. Other diagnostic tests, including measurement of total fecal bile acids and fasting serum 7α-hydroxy-4-cholesten-3-one (C4), a bile acid precursor, show increased fecal losses and synthesis of bile acids in PBAD. (3,4,8-10)

Bile acid synthesis is regulated by the ileal hormone fibroblast growth factor 19 (FGF19) in humans, or by its orthologue FGF15 in rodents. FGF15/19 synthesis is stimulated by absorption of bile acids in the ileum, which act through the principal nuclear bile acid receptor, farnesoid X receptor (FXR) to increase transcription by transactivation of specific target genes through response elements. (11,12) Low FGF15/19 results in increased bile acid synthesis; studies disrupting the FXR-FGF15/19 pathway in animal models have shown increased fecal bile acid loss and chronic diarrhea. The evidence for the role of this system has recently been reviewed. (10) In humans, blood levels of FGF19 and C4 (as a measure of new bile acid synthesis) are inversely correlated and show considerable variation during the day following meals (13), in the healthy population overall, (14) and in patients with IBS. (3,15,16)

Previous studies from our group demonstrated that patients with PBAD have low median fasting serum FGF19 concentrations. (17) We suggested that this would result in overproduction of bile acids, which could exceed the capacity of the terminal ileum to reabsorb them, and would help explain earlier findings of a larger bile acid pool and no impairment of ileal uptake in PBAD. (18-20) We have extended this finding in a larger prospective series of patients with chronic diarrhea, where PBAD patients have significantly lower, but variable, FGF19. (16) Another group has confirmed low FGF19 in IBS patients with SeHCAT less than 10%. (3)

The reasons for these varying reductions in FGF19 are currently unclear. Consequently, the aim of these present studies was to determine whether specific potential mechanisms could be implicated. We performed a series of different investigations in our defined patient population, with bile acid retention defined by SeHCAT retention, to identify associated factors which could affect FGF19

production and action. First, as variable FGF19 patterns during the day following meals have been identified in preliminary studies in our earlier report, we wished to characterize these more thoroughly and define basal and meal-stimulated responses. (17) Secondly, as hypertriglyceridemia has been previously linked to increased bile acid production and absorption, we aimed to determine the extent of this in our PBAD patients. (14,21,22)

We also initiated exploratory studies of certain functional genetic variants, including those which have been identified in the FXR gene (*NR1H4*) (23,24) which might affect FGF19 production. We looked at variants in the *FGF19* gene in the 3'-untranslated region which could affect transcript stability. Other candidate genes were explored where variation could affect intracellular bile acid concentration and FGF19 expression including the ileal bile acid transporters ASBT (*SLC10A2*) and OSTα (*SLC51A*). (25) Although they would not be expected to affect FGF19 serum levels, variations in the FGF19 receptor, *FGFR4*, and its co-receptor, Klotho-β (*KLB*), have been previously been identified in patients with IBS and are related to colonic transit. (4,26,27) These could influence the overall responsiveness to FGF19 by affecting the specific signaling cascade of this hormone. Furthermore, bile acids bind to the cell surface receptor known as TGR5 or GPBAR1 (gene *GPBAR1*), which is expressed on enteric neurons and endocrine cells. TGR5 has been shown to be important for prokinetic actions of bile acids in mice colon, and variants have been associated with human colonic transit, fecal bile acids and IBS-D. (28) Additionally, variants of *TNFSF15* have also been associated with IBS-D and could contribute to the disease mechanism. (29)

Finally, we report separate experiments where we directly determined basal and bile acid-stimulated transcript expression of FGF19 and other key genes in ileal explants obtained from patients with chronic diarrhea with known SeHCAT values. In all these studies, we hypothesized that some of these mechanisms would be abnormal in subgroups of robustly phenotyped patients with PBAD.

METHODS

Patients

Samples from 162 patients with chronic diarrhea were available for study of blood values and genetic variation. 90 of these have been included in a previous report of a prospective series from two sites. (16) Additional patients were studied for ileal gene expression. The composition of patient groups in the different studies is shown in Table1.

All patients had a mean stool number of more than 3 per day, mean stool form on the Bristol stool form scale greater than 5, and a duration of more than 3 months. Patients underwent standard testing including blood tests and colonoscopy or flexible sigmoidoscopy as clinically indicated to exclude other causes of diarrhea such as colorectal neoplasia, coeliac or inflammatory bowel disease. SeHCAT tests were performed as recommended by the manufacturer (GE Healthcare, UK). Patients with 7d SeHCAT retention of <15% were diagnosed as having primary BAD; those with SeHCAT >15% were classed as idiopathic diarrhea controls (ID). Subjects with various secondary causes of BAD, such as Crohn's disease, ileal resection or cholecystectomy, were not included in these studies. Participants gave fully informed consent to be included in research studies as approved by the institutional review board of the Research Ethics Committee of the Hammersmith and Queen Charlottes & Chelsea Hospitals. Fasting blood samples were taken and processed for FGF19, cholesterol and triglycerides and stored for subsequent DNA preparation.

Assays

Serum FGF19 was quantified by a commercially available assay (FGF19 Quantikine ELISA, Cat. No. DF1900; R&D Systems, Minneapolis, MN, USA). Total bile acids were measured by a 3α-hydroxysteroid dehydrogenase assay, and cholesterol and triglycerides were quantified by standard colorimetric techniques in the routine Clinical Chemistry laboratory.

Meal-stimulated FGF19 response

A subgroup of 19 subjects with PBAD were studied to determine post-prandial changes in FGF19 over a six-hour time course. After an overnight fast, blood was sampled every 90 minutes for 6 hours from approximately 09:00 to 15:00. Meals were provided after the first fasting sample at 09:00 (breakfast) and 12:00 (lunch). The composition of the meals was as follows: breakfast, total weight 395g, total energy 2153KJ, protein: carbohydrate: fat energy ratio 9%:71%:20%; lunch, total weight 690g, total energy 2860KJ, protein: carbohydrate: fat energy ratio 11%: 58%: 31%.

Three phenotypes of FGF19 response were identified as an arbitrary classification based on our findings: (i) Low-low (L - L), all 5 serum FGF19 levels <300pg/ml; (ii) Low-high (L - H), sample 1 <200pg/ml, sample 4 or 5 >400pg/ml; (iii) High-high (H - H) samples 1 and 3 or 5 >300pg/ml. FGF19 area under the curve (AUC) values were calculated.

Genetic polymorphisms

In a separate part of the study, genomic DNA was extracted from 75 subjects by standard techniques with PBAD and 86 ID control subjects. Ten different candidate SNPs were analyzed: two

from the gene encoding FGF19 (rs1789170, rs948992), two from FXR (rs61755050, rs56163822), and one each from FGFR4 (rs376618), Klotho-β (rs17618244), ASBT (rs188096), OSTα (rs939885), GPBAR1 (rs11554825) and TNFSF15 (rs7848647). Genotyping of the SNPs was performed using Taqman 5' allelic discrimination assays (Applied Biosystems, Foster City, CA, USA). In exploratory studies we looked at allele frequencies and other genetic models including dominant and recessive genotype differences.

Ileal transcript expression

At routine diagnostic ileo-colonoscopy in 31 patients being investigated for unexplained diarrhea, additional biopsies were taken from the ileal mucosa with informed consent. Colonoscopy was performed after standard bowel preparation and fasting for at least 6h. No colonoscopic or histological abnormalities were detected in these patients. All had SeHCAT test results.

In 15 patients, biopsies were immediately stored in RNAlater for subsequent RNA extraction. These provided basal transcript values. In the other 16 patients, bile acid stimulated transcript expression was investigated in explant culture as previously described. (12) Groups of 2-3 biopsies were incubated separately with either bile acid-free culture media (unstimulated control), or 50µmol/L of chenodeoxycholate (CDCA) or glyco-CDCA (GCDCA). After 6h, biopsies were harvested directly into RNAlater. RNA extraction and cDNA synthesis was performed following standard methods. Expression of FGF19 and other transcripts (including ASBT, IBABP, OSTα, OSTβ, SHP and FXR) were quantified relative to GAPDH by qRT-PCR as previously described. (12)

Statistics

Data were usually analyzed by non-parametric tests including Mann-Whitney U-test to compare medians and Spearman Rank correlation (r_s) to look for associations. The parametric Student's t-test was used to compare normally distributed means. In the genetic association studies and other group studies, proportions were compared with Fisher's exact test with two-tailed p values calculated . A p value of <0.05 was considered significant. No corrections in p values were made for multiple comparisons in these relatively small exploratory studies of SNP polymorphism frequency.

RESULTS

An additional 33 patients with primary BAD (SeHCAT retention < 15%) and 39 ID controls (SeHCAT > 15%) were recruited and added to those previously reported from our institution. (16) These had similar clinical findings to those reported before, with median daily stool frequency of 5/d, Bristol

stool form types of 6.0-6.5, abdominal pain in 57-63%, bloating in 68-74%, fecal incontinence in 36-39% and presence of symptoms for about 2 years before SeHCAT testing, none of which differed significantly between the two groups. The median BMI was higher (27.0 vs. 24.0kg/m^2, p=0.009) in the PBAD patients. Biochemical differences were again as found previously; in particular the median fasting FGF19 in the overall PBAD group was significantly lower than that in the ID controls (148 vs. 235 pg/mL, p=0.0005) In the entire group, FGF19 was positively associated with SeHCAT values (r_s=0.33, p=0.0001), with age (r_s=0.23, p=0.002) and with fasting bile acids (r_s=0.32, p<0.0001). FGF19 and BMI was not significantly related (r_s= −0.11, p=0.09) SeHCAT and BMI were weakly inversely associated (r_s= −0.19, p=0.01) but SeHCAT and bile acids were not (r_s= −0.03, p=0.71).

Associations of FGF19 with lipids

The associations between FGF19 and fasting serum lipids were determined in the patients where these were available. The strongest associations were found between FGF19 and total cholesterol (r_s=0.33, p =0.009, n=62) and LDL cholesterol (r_s=0.34, p=0.01). There was a non-significant association between FGF19 and HDL cholesterol (r_s=-0.14, p=0.27).

Serum triglycerides findings were notable. Higher FGF19 concentrations were associated with higher serum triglycerides (r_s=0.20, p=0.04). As FGF19 correlates overall with SeHCAT retention, it might have been expected that triglycerides would correlate positively with SeHCAT retention. However, there was a significant negative association between triglycerides and SeHCAT retention (r_s=-0.27, p=0.005, n=106, Figure 1). Triglycerides were associated with BMI (r_s=0.35, p=0.0001), and age (r_s=0.29, p=0.001). Subjects with elevated triglycerides had significantly lower SeHCAT retention (median 8.0% vs. 19.5%, p=0.01). Using cutoffs of triglycerides <2.4mmol/L and SeHCAT <15% showed a significant segregation (p=0.017, Fisher's exact test) with most of the subjects with raised triglycerides (13 out of 18) having PBAD with SeHCAT <15%. Furthermore, of the other 5 subjects with triglycerides ≥2.4 mmol/L, 3 out of 5 had SeHCAT retention of under 20%.

In the PBAD group with SeHCAT <15%, those with triglycerides ≥ 2.4 mmol/L had a median FGF19 level which was significantly higher than that in those with low triglycerides (Table 2, p=0.05) and which was similar to the medians found in the ID or healthy control groups. These subjects with elevated triglycerides and low SeHCAT retention may represent a separate subset of about 30% of PBAD with a different underlying etiology.

Meal-stimulated FGF19 responses

We looked at patterns of fasting and meal-stimulated FGF19 to see if we could further define different phenotypes (Figure 2, a - c). Based on these patients and previous limited data, we defined arbitrary values for low (L) and normal high (H) fasting and meal-stimulated values. Although 19 subjects underwent serial FGF19 sampling, two subjects did not match the criteria for the subtypes and are not shown. Of the remaining 17, 8 (47%) were defined as having the L - L response, 5 as L - H and 4 as H - H.

The 8 L - L individuals had significantly lower SeHCAT retention than the L - H group (4.4% vs. 8.1%, $p<0.05$) and the H - H group (7.4%, $p=0.07$). In the L - L group, 6 had SeHCAT retention of 5% or less, with the other 2 having retention of 6.7% and 11.6%. SeHCAT retention in the 5 individuals with the L-H pattern was not significantly different to the 4 subjects with the H - H pattern. There were no significant differences in clinical and biochemical parameters between these different groups of patients.

The FGF19 AUC was lower for those with SeHCAT<5% than those with higher values (median 701 pg/mL·6h vs. 1655 pg/mL·6h, $p=0.023$). SeHCAT retention correlated with the AUC ($p<0.05$, Figure 2d). AUC tended to be associated with fewer bowel motions in 24h ($p=0.13$). A higher FGF19 taken at the 15:00 time point was negatively associated with fewer bowel movements per 24h ($r_s=-0.53$ $p=0.02$).

Serum FGF19 and total bile acids at all the time points were related ($r_s=0.46$, $p<0.0001$). No significant relationship was found between the percentage change in total bile acids and FGF19 at the same point but they strongly correlated with changes in FGF19 90 minutes later ($r_s=0.51$, $p<0.001$). This delay may reflect the time needed for ileal FGF19 production (12) and was similar for all three patterns of response.

There were no significant differences between the L - L, L - H or H - H groups in median fasting triglycerides (2.07, 2.65, 0.77 mmol/L) and there was no correlation between fasting triglycerides and FGF19 AUC. There was however a strong correlation between fasting triglycerides and the percentage increase in FGF19 after breakfast between 09:00 and 10:30 ($r_s=0.59$, $p=0.005$). FGF19 rises more with higher fasting triglyceride levels; consistent with the concept that in those subjects with PBAD associated with hypertriglyceridemia, the pathogenesis is not related to a failure of FGF19 production.

Associations with genetic variants

Exploratory studies were performed to look for major differences in allelic frequencies of specific variants in candidate genes (Table 2). DNA samples from 161 subjects were available but not all genotypes were performed in the entire cohort.

Overall there were no differences in allelic frequency that reached significance at the uncorrected p=0.05 level when a SeHCAT cutoff of 15% was used to separate PBAD patients and idiopathic diarrhea controls. At lower SeHCAT cutoffs, the *FGFR4* SNP rs376618 had borderline significant differences, with the minor C allele being less common. (See Supplemental material for a detailed description.)

We also looked at other models for dominant or recessive genotype effects. The *FGFR4* variant rs376618 had borderline significant differences in genotype frequency in PBAD compared with ID subjects, with genotypes containing the minor C allele less frequent at SeHCAT values of <10% (uncorrected p=0.01) and <5% (p=0.05). Overall, TT subjects had a significantly lower SeHCAT to those in the combined CT/CC group (11 vs. 19, p=0.04). However there was a higher proportion of the C allele in the L – L phenotype compared to the other phenotypes (p=0.05).

The *KLB* rs17618244 variant had borderline significance, with the minor A allele less frequent in PBAD patients (p=0.10 to p=0.12 depending on SeHCAT cutoff values). At the 15% cutoff value, the GG/GA genotypes were more common in the PBAD group (p=0.07).

Basal levels of transcript expression in ileal biopsies

Expression of FGF19 and other relevant transcripts was studied in ileal biopsies obtained at routine diagnostic colonoscopy from patients with chronic diarrhea who also had SeHCAT tests (Figure 3). Basal expression was studied in 15 patients: 8 had PBAD with SeHCAT values <15% (3 to 13%) and 7 were ID controls (values >15%). Two patients (SeHCAT 5% and 13%) were excluded from further analysis as ASBT, SHP and FGF19 transcript values were below detection limits, suggesting a non-ileal source of these biopsies.

SeHCAT values in the 13 patients were significantly associated with FGF19 (r_s=0.59, p=0.03) and with ASBT (r_s=0.49, p=0.04) but not with the other transcripts (Figure 3 A & B). Expression of FGF19 and ASBT were strongly correlated (Figure 3C, r_s=0.88, p=0.0002). Additionally IBABP correlated with OSTα (r_s=0.87, p<0.0001) and IBABP with ASBT (r_s=0.63, p=0.01). FXR and SHP expression were associated

with several genes in related pathways (FGF19, ASBT, IBABP, OSTα and each other) but OSTβ did not correlate with any other transcript (Table 3).

All associations were stronger in the ID controls. In the PBAD group, the associations of FGF19 and ASBT with SeHCAT were absent. Although the correlation of FGF19 and ASBT remained strong, those between FXR and OSTα or IBABP were lost.

Stimulation of FGF19 transcript expression in explants

FGF19 expression was studied in 16 patients with diarrhea in ileal explants cultured for 6h with CDCA , GCDCA or under control conditions. Both bile acids were used to compare any effects that could be related to ASBT uptake of GCDCA compared with more non-specific uptake of CDCA.

FGF19 transcript expression was stimulated in all subjects, with a median of 184-fold induction with CDCA, similar to values found previously in non-diarrheal subjects. (12) The lowest fold changes in FGF19 expression were observed in biopsies from patients with the lowest SeHCAT retention values. Positive correlations were found between SeHCAT retention and the magnitude of the fold change in FGF19 expression stimulated by CDCA (Figure 3D, r_s=0.54, p=0.01, n=16) and by GCDCA (Figure 3E, r_s=0.61, p=0.07, n=7). IBABP, OSTα and SHP transcripts were also significantly stimulated to lesser degrees but ASBT was not affected (Table 4). Apart from FGF19, only the degree of stimulation of IBABP correlated with SeHCAT (p=0.04).

The median stimulation of FGF19 by CDCA was significantly lower in patients with SeHCAT retention <10% (n=5) compared with those >10% (n=11) at 16 vs. 182-fold (p=0.01). Stimulation of IBABP, OSTα and SHP were not significantly different.

DISCUSSION

In patients with PBAD, defined by low SeHCAT retention, we have investigated and shown a variety of mechanisms may be implicated in producing the lower median fasting FGF19 values that we have previously identified. These contributory mechanisms include fasting hypertriglyceridemia and different basal and meal-related responses. We have shown impaired ileal FGF19 transcript expression in the basal state and also impaired bile acid-dependent stimulation. Exploratory studies of possible candidate genetic variation shows only weak associations, which will need confirmation in larger series,

with greater power, but these current findings do indicate there is no large effect from any of the particular functional genetic variants we chose to study.

Our studies indicate that different phenotypes can be defined in patients with PBAD. This may explain the variability we encountered previously in fasting FGF19 serum values, which reduced the predictive value of a single test. (16) The higher FGF19 values found in the patients with fasting hypertriglyceridemia suggest these patients constitute a different subgroup and are in keeping with previous work. Gälman and colleagues studied a large group of over 400 normal subjects without clinical diarrhea, with measurements including FGF19, C4 and triglycerides. (14) Overall they found a negative correlation between FGF19 and corrected C4, and between C4 and triglycerides. In their patients with high C4 above the 95th percentile, 35% had raised triglycerides >2.4mmol/L. This is a similar proportion to that found in our PBAD patients (30%), who would also be expected to have raised C4. (30) Patients with familial hypertriglyceridemia have been shown to have an increased rate of bile acid synthesis and abnormal bile acid absorption has been suggested. (21) These patients have been shown to have reduced expression of ASBT mRNA and protein to about half of control values (22), but no putative causative genetic variant that can explain this has been identified. (31) We have not shown whether these patients with high triglycerides in our cohort with diarrhea have reduced ASBT. It is unclear why they should have essentially normal fasting FGF19 values, but they do appear to constitute a subgroup of about 30% with a markedly altered lipid profile, meriting further study.

We had found preliminary evidence for different fasting and meal-stimulated FGF19 responses in our initial study of PBAD patients (17) and now have defined these patterns more clearly. Almost half the PBAD patients had the L – L pattern, where fasting and post-prandial samples were all below 200pg/mL, which differs from the typical meal-stimulated response in healthy subjects shown previously. (13) The lack of significant FGF19 response resembles a normal fasting pattern, but this L – L pattern is also similar to that which we have recently shown in ileal Crohn's disease. (32) These PBAD patients tend have a more severe phenotype, with a lower SeHCAT, but this pattern can also be found with lesser reductions in SeHCAT. We only studied responses for 6h and a more prolonged study could yield further patterns. Additionally, it is unclear why some patients have the initial high FGF19 of the H – H phenotype; this could be due to prolonged stimulation overnight, lack of fasting or perhaps a stress response related to the demands of the study and venesection.

An impaired hepatic response to FGF19 due to functional genetic variation in components of the receptor pathway (FGFR4, KLB or further downstream) could also result in excessive bile acid synthesis

and high FGF19 levels. Variants in the *FGFR4* and *KLB* genes described by Camilleri and colleagues are particularly interesting in this regard. (26,27) They have been shown to be associated with colonic transit and to interact with each other. Furthermore, interactions have previously been demonstrated for *KLB* variations with *GPBAR1*, which codes for the cell-surface bile acid receptor TGR5 and is likely involved with colonic transit. (33)

We found no overall significant differences for any of the gene variants we studied in allele frequency between the PBAD and ID groups, defined with a SeHCAT cutoff of 15%. We acknowledge these groups are unpowered and type II errors may have occurred. There were borderline significant differences in allelic frequency for *FGFR4* at lower SeHCAT cutoffs and in genotype frequencies for the *FGFR4* and *KLB* variants in the small subgroups, and different SeHCAT median values. It must be borne in mind that we have not corrected for multiple comparisons and so the preliminary, exploratory nature of these results must be stressed. This study does suggest however that these genes represent potential targets in appropriately powered much larger studies in patients with well-defined PBAD. For instance, a study adequately powered (80% at $\alpha<0.05$) to show the difference in proportions we found for the *KLB* variant would need 500 subjects in each group. However, our findings are a reminder that these patients are predicted to have impaired activity in the bile acid-FXR-FGF19 hormonal pathway but will not have low blood FGF19 levels.

The transcript data obtained with ileal biopsies and explant cultures were particularly informative. FGF19 transcripts are significantly lower in basal, unstimulated, fasting samples from patients with lower SeHCAT values. However ASBT transcripts are also lower; if ASBT protein levels are also reduced, it is likely that that this results in reduced bile acid uptake and consequently reduced bile acid/FXR-dependent stimulation of FGF19 transcription. This association of ASBT and FGF19 is the strongest of those found in basal levels, which again have not been corrected for multiple comparisons, so the relevance of the weaker associations is less certain. This data again will require further confirmation but this finding would be relevant to the reduced fasting FGF19 blood levels we have shown.

Incubation with CDCA also gives FGF19 transcript stimulation which is related to SeHCAT values. Unconjugated CDCA, unlike glycine-conjugated GCDCA, is not dependent on transport by ASBT and will produce FXR-mediated transcription in cells not expressing ASBT. (34,35) Conceivably, higher levels of OSTα or IBABP expression would reduce the concentration of intracellular bile acids and the FXR signal, but there is little evidence to support this. (36) The similar relationship of SeHCAT with the stimulation

of IBABP, although this is much lower than FGF19 (as also shown before), (12) suggests the defect in PBAD extends to at least one other FXR-regulated transcripts. Other reasons for reduced FXR activity and FGF19 induction in the ileum are not clear. Inflammation in Crohn's ileitis results in lower FGF19 levels, (32) but the current patients all had normal histological biopsies. Inflammatory cytokines inhibit FXR and FGF15 in mouse intestine (37) and could be implicated. FXR activity is affected by many other pathways; for instance, intestinal peroxisome proliferator-activated receptor α-UDP-glucuronosyl-transferase signaling has recently been proposed. (38) Other transcription factors may regulate FGF15/19 transcription. Vitamin D and A responsiveness has been shown in mice (39) but we found no evidence to support this as being relevant in PBAD patients. (40) Further possible mechanisms include impaired FXR activity due to SIRT1 downregulation (41,42) and the effects of the protein Diet1 on FGF19 production. (43)

Impaired ileal FXR responsiveness is predicted to result in reduced FGF19 production with impaired feedback inhibition of hepatic bile acid synthesis. This increased bile acid synthesis leads to excessive fecal bile acid loss (including loss of SeHCAT) and the production of diarrhea. It is of interest to note however that patients with PBAD are able to respond to more potent FXR agonists such as obeticholic acid. (44) In our recent clinical study, 10 PBAD patients who had low median FGF19 but a range of different response patterns all had some increase in FGF19 with treatment. This resulted in a median FGF19 which was similar to healthy controls, and gave improvement in clinical findings. It will be instructive to perform further large, placebo-controlled studies where the present findings are taken into account and patients are carefully phenotyped, and genetic variants defined, to see if the most responsive patients can be identified.

In conclusion, it appears that several phenotypic patterns of PBAD can be identified. These can result in different patterns of FGF19, which may not be reduced in some patients with hypertriglyceridemia or possibly with certain receptor genetic variants. Patients with severe PBAD, with SeHCAT retention values less than 5%, are more likely to have lower meal-related serum FGF19 levels and lower ileal transcript production has been shown. A fuller characterization of these subgroups and their individual pathophysiological causes is needed in larger studies to expand on our exploratory findings, but if confirmed, an understanding of the different phenotypes of PBAD will help optimize stratified, patient-centered therapy in this understudied condition.

TABLES

Table 1: Details of various studies performed

Study	Patient numbers		
	Total	Primary bile acid diarrhea [a]	Idiopathic diarrhea controls
SeHCAT, FGF19	162	69 (36[b]+33[c])	93 (54[b]+39[c])
Triglycerides	106	47	59
Meal-stimulated response	17	17	--
Genetic polymorphisms	161	75	86
Basal ileal gene expression	15	8	7
Stimulated ileal gene expression	16	5	11

[a] Primary bile acid diarrhea was usually defined as SeHCAT <15% 7 day retention. Some studies also analyzed data at 5% and 10% cutoff values. [b] Patients from the previous report (16); [c] additional patients.

Table 2: FGF19 and SeHCAT in patients with PBAD grouped according to triglycerides

Triglycerides (mmol/L)	n	FGF19 (pg/mL)	P	SeHCAT %	P
≥ 2.4	13	236 ± 49		7.0	
< 2.4	31	150 ± 18	0.05	6.7	NS

44 patients had SeHCAT retention <15% with fasting triglyceride and FGF19 measurements. Values shown are mean ± SEM, and the groups were compared by two-tailed t-test.

Table3: Summary of exploratory genetic studies of variants in candidate genes in PBAD

Gene product	Gene	SNP		Position	Number of subjects		MAF		P
					ID	PBAD	ID	PBAD	
FGF19	**FGF19**	rs1789170	G>A	3'-UTR	84	72	0.357	0.396	0.48
		rs948992	A>G	3'-UTR	86	71	0.366	0.331	0.55
FXR	**NR1H4**	rs61755050	T>C	Met173Thr	60	51	0	0.020	0.21
		rs56163822	G>T	Translation start	80	67	0.044	0.030	0.76
FGFR4	**FGFR4**	rs376618	T>C	Leu136Pro	86	70	0.285	0.221	0.24
KLB	**KLB**	rs17618244	G>A	Arg728Gln	86	71	0.186	0.120	0.12
ASBT	**SCL10A2**	rs188096	C>A	Ala171Ser	86	71	0.098	0.113	0.71
OSTα	**SLC51A**	rs939885	G>A	Val86Ile	55	57	0.482	0.493	0.90
TGR5	**GPBAR1**	rs11554825	T>C	5'-UTR	47	34	0.404	0.426	0.87
TNFSF15	**TNFSF15**	rs7848647	C>T	5'-flanking	87	71	0.282	0.338	0.33

MAF, minor allele frequency; ID, idiopathic diarrhea controls; PBAD, primary bile acid diarrhea. The results shown here are for PBAD and ID patients defined as SeHCAT <15% and >15% respectively (see text).

Table 4: Relationships of basal levels of ileal transcript expression in patients with chronic diarrhea and known SeHCAT values

		All Subjects						
		SeHCAT	**FGF19**	**ASBT**	**IBABP**	**OSTα**	**OSTβ**	**SHP**
FGF19	r_s	0.59						
	p	**0.03**						
ASBT	r_s	0.49	0.88					
	p	**0.04**	**<0.001**					
IBABP	r_s	0.35	0.41	0.63				
	p	0.12	0.11	**0.01**				
OSTα	r_s	0.24	0.32	0.37	0.87			
	p	0.22	0.17	0.10	**<0.001**			
OSTβ	r_s	-0.08	0.18	0.08	-0.11	-0.02		
	p	0.40	0.30	0.40	0.36	0.47		
SHP	r_s	-0.04	0.61	0.53	0.55	0.51	-0.05	
	p	0.44	**0.02**	**0.03**	**0.02**	**0.04**	0.44	
FXR	r_s	0.31	0.67	0.79	0.61	0.43	-0.18	0.54
	p	0.15	**0.01**	**<0.001**	**0.01**	0.07	0.28	**0.03**

Spearman rank correlation coefficients (r_s) and p values are shown for the relationships between expression for all 13 subjects. Significant p values (<0.05) uncorrected for multiple comparisons are shown in bold.

Table 5: Stimulation of ileal transcripts by chenodeoxycholic acid and relationship to SeHCAT

Transcript	Stimulation by CDCA	p	Correlation with SeHCAT	p
FGF19	184	0.0005	0.54	0.01
IBABP	2.2	0.002	0.47	0.04
OSTα	2.8	0.001	-0.10	NS
SHP	2.6	0.002	-0.16	NS
ASBT	1.0	NS	-0.05	NS

Median values for the degree of stimulation (fold-changes) with CDCA 50µmol/L for 6h are shown with p values (Wilcoxon test). The relationships between stimulation and SeHCAT values are shown as Spearman rank correlation coefficients and p values. n=12-16 patients. NS, $p>0.05$.

Table 6: Possible subgroups of bile acid diarrhea based on pathophysiological factors

<table>
<tr><td colspan="5">Bile acid diarrhea
Raised fecal BA
Reduced SeHCAT retention</td></tr>
<tr><td colspan="3">Overproduction
Primary BA Diarrhea</td><td colspan="2">Impaired reabsorption
Bile Acid Malabsorption</td></tr>
<tr><td colspan="2">↓ Feedback inhibition of BA synthesis
(BA-FXR-FGF19-FGFR4/KLB pathway)</td><td rowspan="2">Associated with hypertriglyceridemia</td><td rowspan="2">Secondary to ileal disease / resection</td><td rowspan="2">Secondary to other intestinal disorders</td></tr>
<tr><td>Impaired ileal FGF19 production

↓ Blood FGF19
↓ Baseline FGF19
(associated ↓ ASBT)
± ↓Stimulated FGF19</td><td>Hepatic resistance to FGF19 actions

(? associated with FGFR4/KLB variants)</td></tr>
</table>

FIGURES

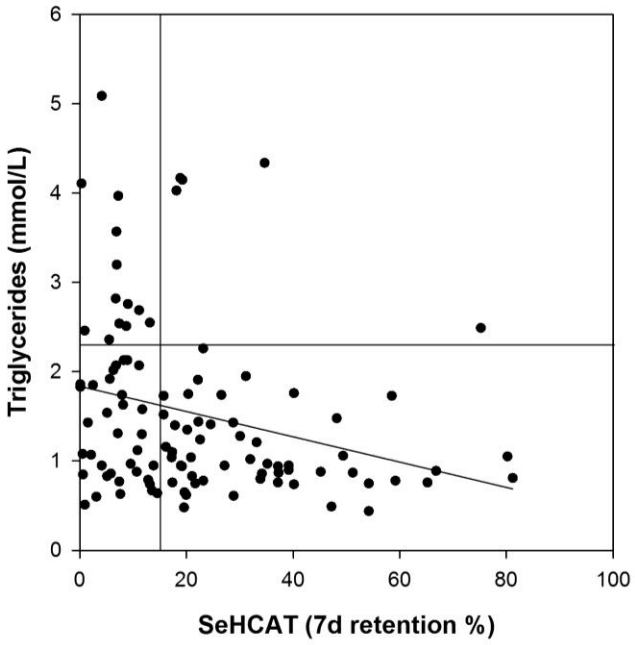

Figure 1 The relationship of fasting triglycerides to SeHCAT 7day retention percentage**.** 106 patients were studied. The linear regression line and values for SeHCAT=15% and for triglycerides=2.4mmol/L are shown.

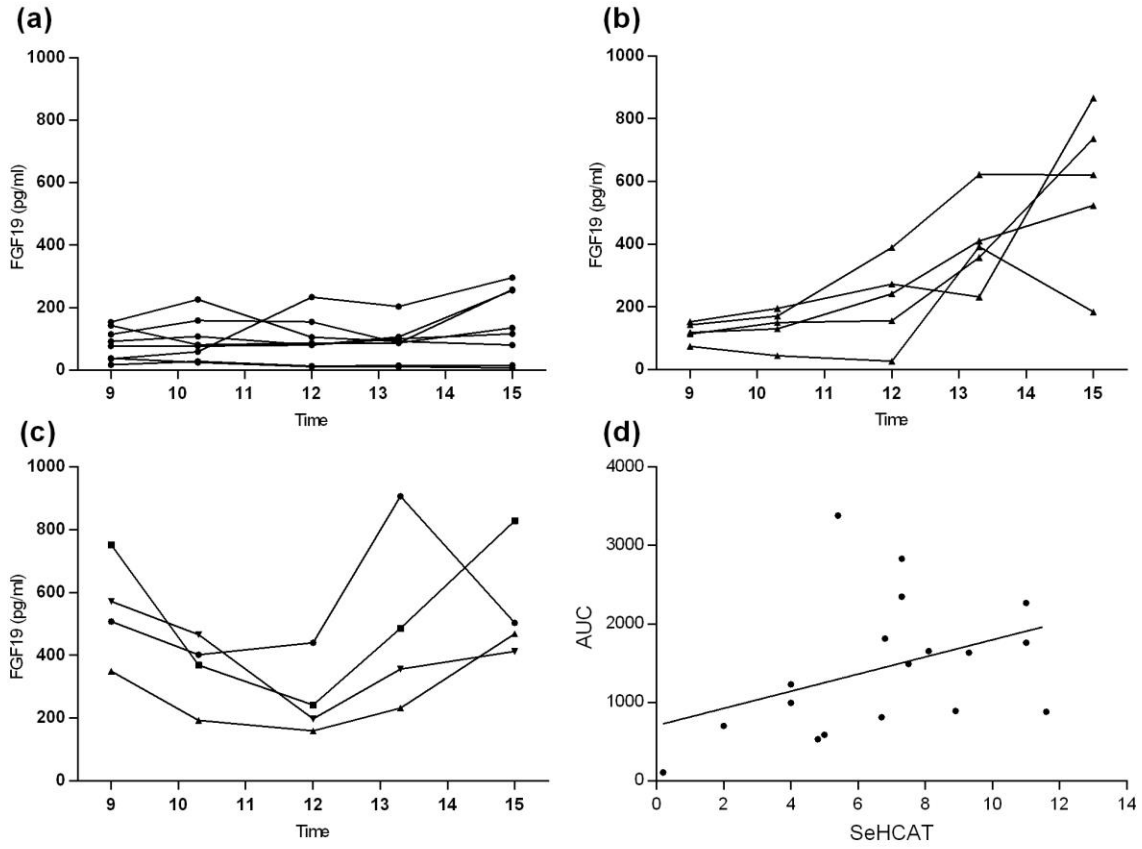

Figure 2 Patterns of serum FGF19 during a 6 hour time-course. Fasting samples were taken at 09:00 (9) and then every 90min until 15:00 (15). Three differing patterns of individual response are shown (see text for definitions): (a) L – L; (b) L – H; (c) H – H. (d) The relationship of the 6h area under the curve (AUC) for FGF19 to the SeHCAT 7d retention is shown for all patients.

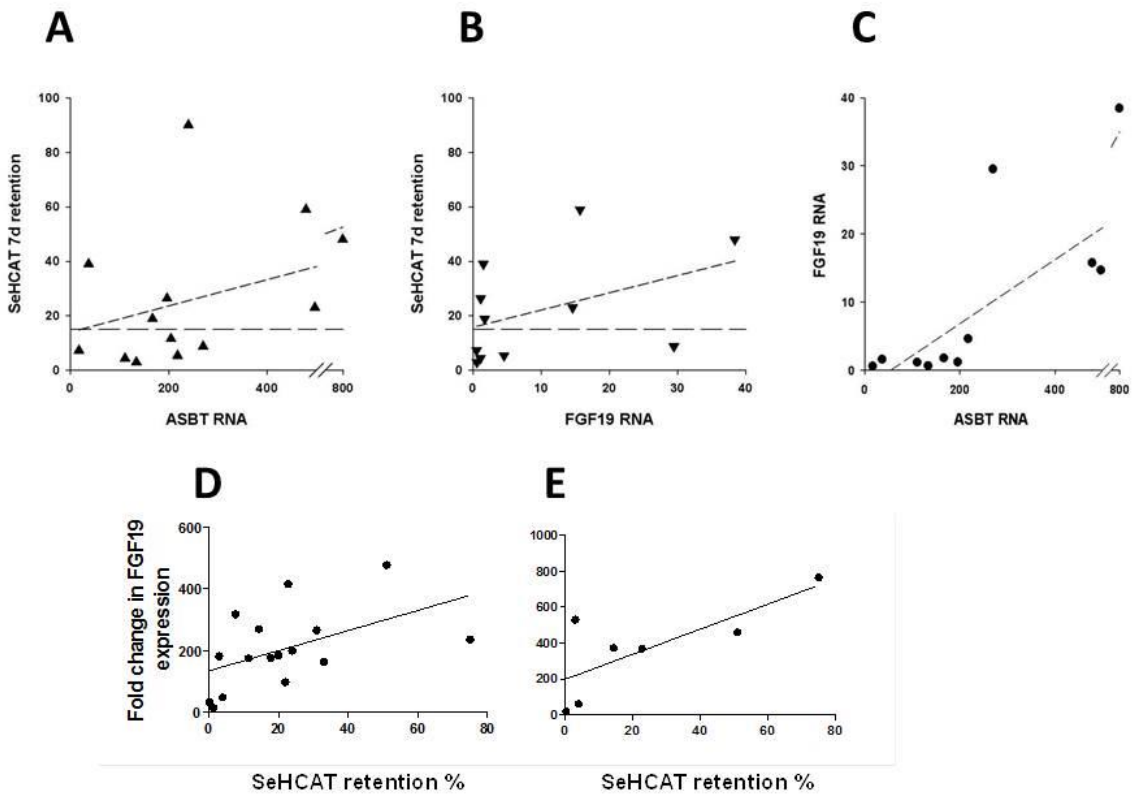

Figure 3 Ileal transcript expression. (A) – (C) are basal RNA expression, (D) and (E) fold-change in stimulated RNA expression. (A) ASBT RNA and relationship to SeHCAT; (B) FGF19 RNA and SeHCAT; (C) FGF19 and ASBT; (D) fold-change in FGF19 expression with CDCA (50µmol/L) and SeHCAT, and (E) fold-change in FGF19 with GCDCA (50µmol/L) and SeHCAT. FGF19 and ASBT values were corrected for GAPDH expression. Linear regression lines are shown. In (A) and (B) lines show SeHCAT values of 15%.

REFERENCES

1. Walters JR. Defining primary bile acid diarrhea: making the diagnosis and recognizing the disorder. Expert Rev Gastroenterol Hepatol 2010;4:561-7.

2. Wedlake L, A'Hern R, Thomas K *et al*. Systematic review: the prevalence of idiopathic bile acid malabsorption (I-BAM) as diagnosed by SeHCAT scanning in patients with diarrhoea-predominant irritable bowel syndrome (IBS). Aliment Pharmacol Ther 2009;30:707-17.

3. Bajor A, Tornblom H, Rudling M *et al*. Increased colonic bile acid exposure: a relevant factor for symptoms and treatment in IBS. Gut 2015;64:84-92.

4. Camilleri M, Busciglio I, Acosta A *et al*. Effect of increased bile acid synthesis or fecal excretion in irritable bowel syndrome-diarrhea. Am J Gastroenterol 2014;109:1621-30.

5. Gracie DJ, Kane JS, Mumtaz S *et al*. Prevalence of, and predictors of, bile acid malabsorption in outpatients with chronic diarrhea. Neurogastroenterol Motil 2012;24:983-e538.

6. Kurien M, Evans KE, Leeds JS *et al*. Bile acid malabsorption: an under-investigated differential diagnosis in patients presenting with diarrhea predominant irritable bowel syndrome type symptoms. Scand J Gastroenterol 2011;46:818-22.

7. Slattery SA, Niaz O, Aziz Q *et al*. Systematic review with meta-analysis: the prevalence of bile acid malabsorption in the irritable bowel syndrome with diarrhoea. Aliment Pharmacol Ther 2015.

8. Brydon WG, Nyhlin H, Eastwood MA *et al*. Serum 7 alpha-hydroxy-4-cholesten-3-one and selenohomocholyltaurine (SeHCAT) whole body retention in the assessment of bile acid induced diarrhoea. Eur J Gastroenterol Hepatol 1996;8:117-23.

9. Vijayvargiya P, Camilleri M, Shin A *et al*. Methods for diagnosis of bile acid malabsorption in clinical practice. Clin Gastroenterol Hepatol 2013;11:1232-9.

10. Walters JR. Bile acid diarrhoea and FGF19: new views on diagnosis, pathogenesis and therapy. Nat Rev Gastroenterol Hepatol 2014;11:426-34.

11. Inagaki T, Choi M, Moschetta A *et al*. Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. Cell Metab 2005;2:217-25.

12. Zhang JH, Nolan JD, Kennie SL *et al*. Potent stimulation of fibroblast growth factor 19 expression in the human ileum by bile acids. Am J Physiol Gastrointest Liver Physiol 2013;304:G940-G948.

13. Lundasen T, Galman C, Angelin B *et al*. Circulating intestinal fibroblast growth factor 19 has a pronounced diurnal variation and modulates hepatic bile acid synthesis in man. J Intern Med 2006;260:530-6.

14. Galman C, Angelin B, Rudling M. Pronounced variation in bile acid synthesis in humans is related to gender, hypertriglyceridaemia and circulating levels of fibroblast growth factor 19. J Intern Med 2011;270:580-8.

15. Wong BS, Camilleri M, Carlson P *et al*. Increased Bile Acid Biosynthesis Is Associated with Irritable Bowel Syndrome with Diarrhea. Clin Gastroenterol Hepatol 2012;10:1009-15.

16. Pattni SS, Brydon WG, Dew T *et al*. Fibroblast growth factor 19 in patients with bile acid diarrhoea: a prospective comparison of FGF19 serum assay and SeHCAT retention. Aliment Pharmacol Ther 2013;38:967-76.

17. Walters JRF, Tasleem AM, Omer OS *et al*. A new mechanism for bile acid diarrhea: defective feedback inhibition of bile acid biosynthesis. Clin Gastroenterol Hepatol 2009;7:1189-94.

18. van Tilburg AJ, de Rooij FW, van den Berg JW *et al*. Primary bile acid diarrhoea without an ileal carrier defect: quantification of active bile acid transport across the ileal brush border membrane. Gut 1991;32:500-3.

19. van Tilburg AJ, de Rooij FW, van den Berg JW *et al*. Primary bile acid malabsorption: a pathophysiologic and clinical entity? Scand J Gastroenterol Suppl 1992;194:66-70.

20. Bajor A, Kilander A, Fae A *et al*. Normal or increased bile acid uptake in isolated mucosa from patients with bile acid malabsorption. Eur J Gastroenterol Hepatol 2006;18:397-403.

21. Angelin B, Hershon KS, Brunzell JD. Bile acid metabolism in hereditary forms of hypertriglyceridemia: evidence for an increased synthesis rate in monogenic familial hypertriglyceridemia. Proc Natl Acad Sci U S A 1987;84:5434-8.

22. Duane WC, Hartich LA, Bartman AE *et al*. Diminished gene expression of ileal apical bile acid transporter explains impaired absorption of bile acid in patients with hypertriglyceridemia. J Lipid Res 2000;41:1384-89.

23. Van Mil SW, Milona A, Dixon PH *et al*. Functional variants of the central bile acid sensor FXR identified in intrahepatic cholestasis of pregnancy. Gastroenterology 2007;133:507-16.

24. Attinkara R, Mwinyi J, Truninger K *et al*. Association of genetic variation in the NR1H4 gene, encoding the nuclear bile acid receptor FXR, with inflammatory bowel disease. BMC Res Notes 2012;5:461.

25. Dawson PA, Lan T, Rao A. Bile acid transporters. J Lipid Res 2009;50:2340-57.

26. Rao AS, Wong BS, Camilleri M *et al*. Chenodeoxycholate in females with irritable bowel syndrome-constipation: a pharmacodynamic and pharmacogenetic analysis. Gastroenterology 2010;139:1549-58, 1558.

27. Wong BS, Camilleri M, Carlson PJ *et al*. A Klotho-beta Variant Mediates Protein Stability and Associates with Colon Transit in Irritable Bowel Syndrome with Diarrhea. Gastroenterology 2011;140:1934-42.

28. Camilleri M, Shin A, Busciglio I *et al*. Genetic variation in GPBAR1 predisposes to quantitative changes in colonic transit and bile acid excretion. Am J Physiol Gastrointest Liver Physiol 2014;307:G508-G516.

29. Swan C, Duroudier NP, Campbell E *et al*. Identifying and testing candidate genetic polymorphisms in the irritable bowel syndrome (IBS): association with TNFSF15 and TNFalpha. Gut 2013;62:985-94.

30. Pattni SS, Brydon WG, Dew T *et al*. Fibroblast Growth Factor 19 and 7α-hydroxy-4-cholesten-3-one in the diagnosis of patients with possible bile acid diarrhea. Clin Trans Gastroenterol 2012;3:e18.

31. Love MW, Craddock AL, Angelin B *et al*. Analysis of the ileal bile acid transporter gene, *SLC10A2*, in subjects with familial hypertriglyceridemia. Arterioscler Thromb Vasc Biol 2001;21:2039-45.

32. Nolan JD, Johnston IM, Pattni SS *et al*. Diarrhea in Crohn's Disease: investigating the role of the ileal hormone Fibroblast Growth Factor 19. J Crohns Colitis 2015;9:125-31.

33. Camilleri M, Shin A, Busciglio I *et al*. Genetic variation in GPBAR1 predisposes to quantitative changes in colonic transit and bile acid excretion. Am J Physiol Gastrointest Liver Physiol 2014;307:G508-G516.

34. Makishima M, Okamoto AY, Repa JJ *et al*. Identification of a nuclear receptor for bile acids. Science 1999;284:1362-5.

35. Parks DJ, Blanchard SG, Bledsoe RK *et al*. Bile acids: natural ligands for an orphan receptor. Science 1999;284:1365-8.

36. Balesaria S, Pell RJ, Abbott LJ *et al*. Exploring possible mechanisms for primary bile acid malabsorption: evidence for different regulation of ileal bile acid transporter transcripts in chronic diarrhoea. Eur J Gastroenterol Hepatol 2008;20:413-22.

37. Gadaleta RM, van Erpecum KJ, Oldenburg B *et al*. Farnesoid X receptor activation inhibits inflammation and preserves the intestinal barrier in inflammatory bowel disease. Gut 2011;60:463-72.

38. Zhou X, Cao L, Jiang C *et al*. PPARalpha-UGT axis activation represses intestinal FXR-FGF15 feedback signalling and exacerbates experimental colitis. Nat Commun 2014;5:4573.

39. Schmidt DR, Holmstrom SR, Fon TK *et al*. Regulation of bile acid synthesis by fat-soluble vitamins A and D. J Biol Chem 2010;285:14486-94.

40. Johnston IM, Pattni SS, Nolan JD *et al*. Vitamins A, D amd B12 in patients with primary bile acid diarrhea: a prospective study. Dig Dis Sci 2015;submitted.

41. Garcia-Rodriguez JL, Barbier-Torres L, Fernandez-Alvarez S *et al*. SIRT1 controls liver regeneration by regulating bile acid metabolism through farnesoid X receptor and mammalian target of rapamycin signaling. Hepatology 2014;59:1972-83.

42. Kazgan N, Metukuri MR, Purushotham A *et al*. Intestine-specific deletion of SIRT1 in mice impairs DCoH2-HNF-1alpha-FXR signaling and alters systemic bile acid homeostasis. Gastroenterology 2014;146:1006-16.

43. Vergnes L, Lee JM, Chin RG *et al*. Diet1 functions in the FGF15/19 enterohepatic signaling axis to modulate bile acid and lipid levels. Cell Metab 2013;17:916-28.

44. Walters JR, Johnston IM, Nolan JD *et al*. The response of patients with bile acid diarrhoea to the farnesoid X receptor agonist obeticholic acid. Aliment Pharmacol Ther 2015;41:54-64.

SUPPLEMENTAL MATERIAL

Characterizing Factors Associated with Differences in FGF19 Blood Levels and Synthesis in Patients with Primary Bile Acid Diarrhea

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Results: Further associations with genetic variants

A fuller description of the allelic and genotypic variants that were studied and the associations that were found is detailed below.

FGF19

For the two SNPs in the 3'-UTR of the *FGF19* gene, no significant differences between the patient groups for allelic frequency and no dominance effects were found.

FXR

The FXR variant rs61755050 produces a non-synonymous change (Met173Thr) and has previously been shown to affect FXR function. (1) Two subjects out of 111 successful genotypes were heterozygotes; both had severe PBAD (SeHCAT 3% and 2%), low serum FGF19 (59 and 149 pg/mL) with total fasting bile acids of 0.9 and 5.9 mmol/L respectively. This result was not significant due to small sample size; power calculations for a significance of 0.05 and a power of 0.80 suggest around 500 subjects would be required. The other FXR variant, rs56163822, which produces a change at the

translation start site and has been associated with IBD and intrahepatic cholestasis of pregnancy, (1,2) has a low MAF and no significant difference was found with these small groups. One individual was homozygous for the minor allele but had ID with a SeHCAT value of 21% and a fasting FGF19 237 pg/mL.

FGFR4

The *FGFR4* variant rs376618 has been linked to faster colonic transit in TT homozygotes. (3) We found no significant difference in MAF at a SeHCAT cut-off of 15%, but at lower cutoff values of 10% and 5%, the minor C allele was less frequent in PBAD than in ID patients, with uncorrected p values of 0.0127 and 0.0507 (Supplemental table 1).

When genotypes were looked at, there was a lower proportion of CT heterozygotes in PBAD compared with in ID subjects (24% vs. 43%) and CC + CT (i.e. C dominant) genotypes were also significantly less frequent compared to the TT genotype (p = 0.053). Proportions were significant at SeHCAT cutoffs of 10% and 5%. TT subjects have a significantly lower SeHCAT to those in the combined CT + CC group (11% vs. 19%, p = 0.04) but FGF19 levels were not significantly different (214 vs. 178 pg/mL, p = 0.33).

Supplemental Table 1: Analysis of *FGFR4* SNP rs rs376618 at different SeHCAT values

SeHCAT cutoff value	Minor allele frequency			Genotype dominance model	
	C			T	C
	ID	PBAD	p	p	p
15%	0.285	0.221	0.241	0.567	0.053
10%	0.300	0.167	0.013	0.548	0.009
5%	0.279	0.140	0.051	0.694	0.047

Idiopathic diarrhea (ID) patients have SeHCAT values greater than the cutoff and PBAD patients less than the cutoff value.

KLB

The rs17618244 SNP in the *KLB* gene has previously been shown to be associated with colonic transit in IBS with the G allele also linked to faster gut transit time in response to oral chenodeoxycholate acid. (3,4) The G allele was more frequent in the PBAD group than in the ID group with p values about 0.1 for different SeHCAT cutoffs (see Supplemental Table 2).

The GG or GA genotypes were more common than the AA genotype but with only borderline significance at the SeHCAT cutoff of 15% (p = 0.07). Of the 8 subjects with the genotype AA, 7 had ID (SeHCAT ranging from 19 – 51 %), and only 1 had PBAD (SeHCAT 7%).

Supplemental Table 2: Analysis of *KLB* SNP rs17618244 at different SeHCAT values

SeHCAT cut-off value	Minor allele frequency			Genotype dominance model	
	A			G	A
	ID	PBAD	p	p	p
15%	0.188	0.120	0.118	0.072	0.365
10%	0.183	0.106	0.098	0.270	0.181
5%	0.173	0.077	0.096	0.353	0.224

Idiopathic diarrhea (ID) patients have SeHCAT values greater than the cutoff and PBAD patients less than the cutoff value.

ASBT

The rs188096 SNP in the *SCL10A2* gene, encoding Ala171Ser in ASBT, has been found to be present in a family with congenital PBAD (5) and has been previously studied in larger groups of patients. (6) No significant difference was found here although the sample size is low. Three individuals had the AA genotype, one with ID (SeHCAT 37%) and two with severe PBAD (SeHCAT <2%). All 3 individuals were clinically otherwise unremarkable. The ID individual had an unremarkable FGF19 of 238 pg/mL and the two PBAD individuals also had atypical high FGF19 (260 and 313 pg/mL).

OSTα

The rs939885 SNP in OSTα had similar allelic frequencies in the two groups.

GPBAR1

The rs11554825 SNP in the 5'-UTR of the GPBAR1 gene has been associated with colonic transit and fecal bile acids in IBS patients (7) but we found no differences in allelic or genotype frequencies between our patient groups.

TNFSF15

Although TNFSF15 is not involved with bile acids, SNPs including rs7848647 have been associated with post-infectious IBS and could have been increased in our ID controls. (8) No significant difference was found in our patient groups.

Relationships with FGF19 phenotypic variation

In the 17 subjects where FGF19 was profiled throughout the day, allelic frequencies were compared between the L - L phenotype and the combined group of the other phenotypes. The FGFR4 SNP rs376618 showed a significantly higher proportion of the C allele in the L - L phenotype compared with the others (0.375 vs. 0.091, $p = 0.05$). The ASBT SNP rs188096 has a higher proportion of the A allele in the L-L phenotype (0.250 vs. 0.091, $p = 0.22$), and the FGF19 SNP rs948992 had a lower proportion of the G allele in the L - L group than the rest (0.188 vs 0.455, $p = 0.17$), but neither difference reached significance.

Specific references

1. Van Mil SW, Milona A, Dixon PH *et al*. Functional variants of the central bile acid sensor FXR identified in intrahepatic cholestasis of pregnancy. Gastroenterology 2007;133:507-16.

2. Attinkara R, Mwinyi J, Truninger K *et al*. Association of genetic variation in the NR1H4 gene, encoding the nuclear bile acid receptor FXR, with inflammatory bowel disease. BMC Res Notes 2012;5:461.

3. Rao AS, Wong BS, Camilleri M *et al*. Chenodeoxycholate in females with irritable bowel syndrome-constipation: a pharmacodynamic and pharmacogenetic analysis. Gastroenterology 2010;139:1549-58, 1558.

4. Wong BS, Camilleri M, Carlson PJ *et al*. A Klotho-beta Variant Mediates Protein Stability and Associates with Colon Transit in Irritable Bowel Syndrome with Diarrhea. Gastroenterology 2011;140:1934-42.

5. Oelkers P, Kirby LC, Heubi JE *et al*. Primary bile acid malabsorption caused by mutations in the ileal sodium-dependent bile acid transporter gene (SLC10A2). J Clin Invest 1997;99:1880-7.

6. Montagnani M, Love MW, Rössel P *et al*. Absence of dysfunctional ileal sodium-bile acid cotransporter gene mutations in patients with adult-onset idiopathic bile acid malabsorption. Scand J Gastroenterol 2001;36:1077-80.

7. Camilleri M, Shin A, Busciglio I *et al*. Genetic variation in GPBAR1 predisposes to quantitative changes in colonic transit and bile acid excretion. Am J Physiol Gastrointest Liver Physiol 2014;307:G508-G516.

8. Swan C, Duroudier NP, Campbell E *et al*. Identifying and testing candidate genetic polymorphisms in the irritable bowel syndrome (IBS): association with TNFSF15 and TNFalpha. Gut 2013;62:985-94.